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Promotion of Organophosphate-Induced Delayed Polyneuropathy by Phenylmethanesulfonyl Fluoride[1]

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Promotion of Organophosphate-Induced Delayed Polyneuropathy by Phenylmethanesulfonyl Fluoride. LOTTI, M., CAROLDI, S., CAPODICASA, E., AND MORETTO, A. (1991). *Toxicol. Appl. Pharmacol.* **108**, 234–241. Certain sulfonates, like phenylmethanesulfonyl fluoride (PMSF), carbamates, and phosphinates, when given prior to neuropathic doses of organophosphates such as diisopropyl phosphorofluoridate (DFP), protect hens from organophosphate-induced delayed polyneuropathy (OPIDP). Protection was related to inhibition of the putative target of OPIDP, which is called Neuropathy Target Esterase (NTE). NTE inhibition above 70–80% in the nervous system of hens followed by a molecular rearrangement called aging initiates OPIDP. PMSF and other protective chemicals inhibit NTE but OPIDP does not develop because aging cannot occur. DFP (1 mg/kg sc) inhibited NTE above 70–80% in peripheral nerve and caused OPIDP in hens. Lower doses (0.3 and 0.5 mg/kg sc) caused about 40–60% NTE inhibition and no or marginal OPIDP. Chlorpyrifos (90 mg/kg po) also caused OPIDP. When repeated (30 mg/kg sc daily for 9 days) or single (5–120 mg/kg sc) doses of PMSF were given after either DFP or chlorpyrifos, OPIDP developed in birds treated with nonneuropathic doses of DFP and was more severe in birds treated with chlorpyrifos or higher doses of DFP. PMSF increased NTE inhibition to >90%. Promotion of OPIDP with a single dose of PMSF (120 mg/kg sc) was obtained in birds up to 11 days after a marginally neuropathic dose of DFP (0.5 mg/kg sc). Promotion was also obtained with phenyl *N*-methyl *N*-benzyl carbamate (40 mg/kg iv) but not with non-NTE inhibitors *in vivo* such as paraoxon or benzenesulfonyl fluoride when given at maximum tolerated doses. These results indicate that protection from OPIDP is only one effect of PMSF because promotion of OPIDP is also observed depending upon the sequence of dosing. Either effect is always related to the doses of PMSF, which inhibit NTE.

Organophosphate-induced delayed polyneuropathy (OPIDP) is a rare toxicity caused by certain organophosphorus esters (OPs) in several species and is characterized by axonal degeneration of some long axons in the central and peripheral nervous system (Johnson, 1982, 1987; Lotti *et al.*, 1984; Lotti, 1987a).

Twenty-one years ago it was observed that certain carbamates protect from OPIDP caused by diisopropyl phosphorofluoridate (DFP) (Johnson and Lauwerys, 1969). Other biochemically related chemicals such as phenylmethanesulfonyl fluoride (PMSF) and some phosphinates were also found to be protective when given prior to DFP and other neuropathic OPs (Johnson, 1974). Protection was related to the inhibition of an enzyme in the nervous tissue, Neuropathy Target Esterase (NTE), which is regarded as the molecular target of OPIDP (Johnson, 1982). It is thought that OPIDP is initiated by an intramolecular rearrangement of the phosphorylated NTE (aging) which occurs with certain inhibitors only. Phosphates such as DFP initiate OPIDP

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because aging is chemically possible. Protective inhibitors do not cause OPIDP because inhibited NTE does not age and protect from OPIDP by occupying the target. These molecular changes, usually measured within hours after dosing, correlate with either clinical response 2 weeks later. Little is known about the pathophysiology linking molecular initiation and clinical expression of OPIDP (Moretto *et al.*, 1987) and nothing is known about the functions of this protein, which carries a catalytic activity that is apparently nonessential to the health of neurons. Nevertheless measurement of NTE inhibition (and sometimes aging) is currently exploited to assess changes at the target and to predict the clinical responses produced by such compounds (Lotti, 1990).

In experimental animals (usually the adult hen) initiation of and protection from OPIDP have been related to more than 70% inhibition/aging and to 30–40% inhibition of NTE, respectively, in the target axons (Johnson, 1982; Caroldi *et al.*, 1984; Lotti *et al.*, 1987). When hens were treated with a single oral dose of chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate), NTE inhibition increased from 10% on Day 1 to 70–80% on Day 6, and correlated with OPIDP 2–3 weeks later (Lotti, 1987b). This unusually delayed biochemical response, probably reflecting slow disposal of chlorpyrifos, suggested a "therapeutic" use of PMSF. Several doses of PMSF were then given to hens at daily intervals after chlorpyrifos in order to prevent slow NTE phosphorylation by chlorpyrifos with a substantial and rapid process of sulfonylation. Surprisingly, hens treated with chlorpyrifos plus PMSF were more severely affected than those treated with chlorpyrifos only.

In this paper, we present observations that PMSF and phenyl *N*-methyl *N*-benzyl carbamate promote OPIDP when given after OPs, in addition to their protective effect when given before. Our structure–activity relationship studies show so far that only NTE inhibitors are able to promote OPIDP.

MATERIALS AND METHODS

Chemicals. Chlorpyrifos was a gift from Dow Chemical Co. (Midland, MI). DFP, PMSF, and glycerol formal were purchased from Fluka AG Chem. Fabrik (Buchs, Switzerland). Benzenesulfonyl fluoride (PSF) and atropine sulfate were purchased from Aldrich Chemie (Steinheim, FRG). Phenyl *N*-methyl-*N*-benzylcarbamate was purchased from R. Mattocks (Carshalton, UK). Phenyl valerate and mipafox (*N,N*-diisopropyl phosphorodiamidofluoridate) were purchased from Lark Enterprises (Webster MA). Paraoxon (*O,O* diethyl *p*-nitrophenyl phosphate) was purchased from Sigma Chemical Co. (St. Louis, MO) and purified according to Johnson (1977). Pralidoxime methylsulfate (2-PAM) was a gift from Carlo Erba (Milano, Italy).

Animals. Randomly bred adult hens (1.7–3.0 kg body wt) were caged in groups of 5–10 and allowed food and water *ad libitum*. The animals were randomly divided into groups and treated with the test compound(s) or vehicle. DFP, paraoxon, PMSF, and PSF were dissolved in glycerol formal immediately before use and injected subcutaneously (sc, one or multiple injections) in the anterothoracic region in a total maximal volume of 2.4 ml. Chlorpyrifos was given in glycerol formal by gavage (<1.5 ml of volume). Phenyl *N*-methyl *N*-benzyl carbamate (PMBC) was dissolved in saline immediately before use and injected intravenously (iv) under the wing in a maximal volume of 0.3 ml. Control animals were given equivalent volumes of either glycerol formal or saline. Animals dosed with chlorpyrifos, DFP (1 or more mg/kg sc), or paraoxon were treated with atropine (20 mg/kg ip) to reduce cholinergic toxicity. Animals dosed with chlorpyrifos, in addition to atropine, were also treated with 2-PAM (100 mg/kg ip) twice daily, for up to 10 days after treatment.

Animals were killed by decapitation at chosen times. Brain, lumbosacral spinal cord, and sciatic nerve were excised immediately and either placed in ice-cold 50 mM Tris–HCl buffer (pH 8.0 at 23°C) containing 0.2 mM EDTA and assayed for NTE or stored at −80°C prior to assay.

The clinical evaluation was blindly performed on each bird daily. Walking performance was evaluated according to a 8-point scale as follows: 0 = no defects in posture, standing ability, and walking performance, 1 = minor changes in walking performance, 2 = clear changes in walking performance with some falling of the hind end, 3 = walking limited to short steps with frequent falling of the hind end, 4 = still able to walk but often birds remain sitting on metatarsus, 5 = unable to walk, sitting on metatarsus but able to stand when lifted, 6 = unable to stand when lifted, 7 = unable to stand, wings drop, 8 = unable to stand, extended legs and wings.

Biochemistry. NTE activity in brain and spinal cord was assayed according to Johnson (1977); NTE activity in sciatic nerve was assayed according to Caroldi and Lotti (1982) as modified by Moretto *et al.* (1989). Percentages

of inhibition were calculated from the activities of corresponding tissues obtained from control birds treated with vehicle. NTE activities of controls were always within the range of historical values from our laboratory. (NTE activity ranging from 2.0 to 2.5 in brain, from 0.5 to 0.7 in the spinal cord, and from 0.1 to 0.15 in the peripheral nerve (μmol/min/g of tissue).

Statistics. To verify a normal distribution of clinical scores in small groups of birds is difficult. Furthermore scores are based on descriptive records. Therefore the following nonparametric tests were selected: the Mann–Whitney *U* test to assess the difference between two independent samples, and corrected when one group exceed 20 birds and the Kruskal–Wallis test when more than two samples were compared.

RESULTS

The promoting effect of PMSF on birds pretreated with different OPs is shown in Table 1. Birds treated with marginally neuropathic doses of either chlorpyrifos or DFP became grossly ataxic when nine daily doses of PMSF followed the initial treatment. A similar effect was also obtained with a single higher dose of PMSF. On the contrary, when birds were given paraoxon at highly toxic doses (cholinergic symptoms) and then treated with the same high dose of PMSF they did not become ataxic.

Neuropathology was performed on several central and peripheral nervous tissues from birds in which OPIDP was promoted and indicated that lesions were not qualitatively different from those observed in typical OPIDP (M. Fiori, FIDIA Research Labs, Abano, PD, Italy, personal communication).

Dose–response relationships of initiator (DFP) and promoter (PMSF) on biochemical and clinical endpoints are reported in Table 2. Inspection of the table reveals that the minimal initiating dose of DFP when promotion was provided afterwards (PMSF 120 mg/kg sc) was 0.3 mg/kg sc. With this initiating dose alone, NTE was 40–50% inhibited and not correlated with OPIDP. If PMSF was then given, NTE inhibition rose to >90% and birds became ataxic 2 weeks later. By increasing the dose of DFP, NTE inhibition reached the threshold for OPIDP and animals became marginally affected. When PMSF was then given, NTE inhibition rose again to >90% and birds became grossly ataxic. By further increasing the dose of DFP (1 mg/kg sc) and

TABLE 1

EFFECTS OF PMSF WHEN GIVEN AFTER OPS

Treatment		
OP	PMSF	Ataxia score[a]
Chlorpyrifos 90 mg/kg po	Vehicle	1.3 ± 2.1 (7)
Vehicle	30 mg/kg daily × 9 days sc	0, 0, 0, 0, 0
Chlorpyrifos 90 mg/kg po	30 mg/kg daily × 9 days[b] sc	5.1 ± 2.0 (7)[c]
DFP 0.5 mg/kg sc	Vehicle	0.8 ± 1.2 (26)[d]
Vehicle	120 mg/kg sc[e]	0, 0
DFP 0.5 mg/kg sc	30 mg/kg daily × 9 days[b] sc	5.4 ± 1.7 (5)[d]
DFP 0.5 mg/kg sc	120 mg/kg sc[f]	5.8 ± 1.5 (13)[d]
Paraoxon 0.6 mg/kg sc	120 mg/kg sc[f]	0, 0, 0, 0

[a] Mean ± SD (*n*) or individual animals. Maximal scores are reported and were reached within 5 weeks after initial dosing.

[b] Beginning 24 hr after OP dose.

[c] Different from the group treated with chlorpyrifos only. $p < 0.05$, Mann–Whitney test.

[d] $p < 0.001$, Kruskal–Wallis test.

[e] No ataxia when PMSF was given up to 480 mg/kg.

[f] Given 24 hr after OP.

TABLE 2

BIOCHEMICAL AND CLINICAL EFFECTS OF COMBINED TREATMENT WITH DFP AND PMSF (DOSE–RESPONSE RELATIONSHIP)

Initial treatment DFP (mg/kg sc)	Subsequent treatment 24 hr later PMSF (mg/kg sc)	Ataxia score[a]
0.15	120[b]	0, 0, 0, 0, 0
0.3[c]	Vehicle	0, 0
0.3	120[d]	1.3 ± 1.0 (4)
0.5[e]	Vehicle	0.8 ± 1.2 (26)[f]
0.5	5[g]	3.2 ± 3.3 (5)[f]
0.5	30	3.3 ± 1.5 (4)[f]
0.5	120[h]	5.8 ± 1.5 (13)[f]
1	Vehicle	4.5 ± 2.9 (4)
1	120	8, 8, 8

[a] Mean ± SD (n) or individual animals. Maximal scores are reported and were reached within 5 weeks after the initial dosing. No significant differences were detected among groups as far as the time of both onset and peak of ataxia.

[b] When PMSF was given alone, it caused 93 ± 1% NTE inhibition in brain (n = 4). Percentages in this table were calculated from the mean values of 15 concurrent controls. They were within the range of historical controls as reported under Materials and Methods.

[c] Brain NTE was measured 24 hr after DFP in two animals and was found to be inhibited by 44 and 51%.

[d] Brain NTE was measured 24 hr after PMSF and was found to be inhibited by 95 ± 1% (mean ± SD, n = 4).

[e] Brain, spinal cord, and peripheral nerve NTE levels when measured 24 hr after DFP were found to be inhibited by 81 ± 8, 84 ± 15, and 68 ± 15%, respectively (mean ± SD, n = 5).

[f] Kruskal–Wallis test: Significance $p < 0.001$.

[g] When PMSF was given alone it caused 50 and 47% inhibition of NTE in the brain of two animals.

[h] Brain and peripheral nerve NTE were measured 24 hr after PMSF and found to be inhibited by 92 ± 2% and 92 ± 5% respectively (mean ± SD, n = 3).

then providing PMSF promotion all animals became paralyzed, reaching the maximal clinical score.

The minimal promoting dose of PMSF when tested on birds pretreated with DFP (0.5 mg/kg sc) was found to be 5 mg/kg sc, which caused about 50% NTE inhibition when given to naive animals.

Other esterase inhibitors have been tested for promoting effects and results are reported in Table 3. PMBC caused high inhibition of NTE (≈90%) when given at 40 mg/kg iv to control animals. As expected even this high NTE inhibition was not correlated with OPIDP. However, when PMBC was given after DFP (0.5 mg/kg sc) it promoted ataxia. Also when DFP was followed by both PMBC and PMSF, OPIDP promotion was observed. On the contrary, when two chemicals which do not inhibit NTE, such as PSF (an analog of PMSF) and paraoxon, were given after DFP, no promotion was observed.

Promotion was also observed when the promoting dose of PMSF was delayed up to 11 days after DFP as shown in Table 4.

In order to ascertain whether initial protection by PMSF would overcome the promoting effects of subsequent doses of PMSF, the same inhibitor was given before and after a very large dose of DFP and results are reported in Table 5. It seems that promotion might still occur, at least in part, when animals have been previously protected, even though there is no relationship between the PMSF dose and the promotion effect.

DISCUSSION

Figure 1 summarizes the current knowledge of OPIDP mechanisms. The results of our study show particularly that OPIDP is promoted by doses of PMSF which inhibit NTE, only when NTE has already been partially inhibited by DFP (or chlorpyrifos). Other NTE inhibitors, such as PMBC or phenyl di-*n*-pentyl-phosphinate (15 mg/kg sc after DFP 0.4 mg/kg sc, M.K. Johnson, MRC Toxicology Unit, Carshalton, UK, personal communication), promoted OPIDP at doses which inhibit NTE. Nonneuropathic and nonprotective esterase inhibitors such as paraoxon and benzenesulfonyl fluoride neither inhibited NTE at maximum tolerated doses nor promoted OPIDP. Similar promotion experiments were also negative in chicks (about 40 days of age,

TABLE 3

CLINICAL EFFECTS OF ESTERASE INHIBITORS GIVEN 24 hr APART ON BIRDS PRETREATED WITH MARGINALLY NEUROPATHIC DOSES OF DFP

First dosing	Second dosing	Third dosing	Ataxia score[a]
DFP 0.5 mg/kg sc	Vehicle	Vehicle	0.8 ± 1.2 (26)
Vehicle	PMBC[b] 40 mg/kg iv	Vehicle	0, 0, 0
DFP 0.5 mg/kg sc	PMBC 40 mg/kg iv	Vehicle	5.8 ± 2.5 (5)[c]
DFP 0.5 mg/kg sc	PBMC 40 mg/kg iv	PMSF 120 mg/kg sc	7.2 ± 1.3 (5)
Vehicle	PSF[d] 120 mg/kg sc	—	0, 0, 0
DFP 0.5 mg/kg sc	PSF[e] 120 mg/kg sc	—	0.6 ± 0.5 (5)[f]
DFP 0.3 mg/kg sc	Paraoxon[g] 0.35 mg/kg sc	—	0, 0

[a] Mean ± SD (*n*) or individual animals. Maximal scores are reported and were reached within 5 weeks after initial dosing. No significant difference were detected among groups as far as the time of both onset and peak of ataxia.

[b] The percentage of NTE inhibition was 90 and 84% in the brain of two paired dosed birds at 5 hr after treatment.

[c] Different from birds treated with DFP only. $p < 0.002$, Mann–Whitney test, modified for large samples.

[d] No NTE inhibition was detected in brain of one paired dosed bird, 24 hr after treatment.

[e] The percentage of NTE inhibition 24 hr after dosing was 71 ± 6 in brain and 82 ± 9 in peripheral nerve ($n = 5$).

[f] Not significantly different from birds treated with DFP only. Mann–Whitney test.

[g] When DFP (0.3 mg/kg sc) plus paraoxon (0.6 mg/kg sc) was given, all animals died.

data not shown), which are known to be resistant to OPIDP (Johnson and Barnes, 1970). Finally OPIDP promotion by single doses of PMSF occurred even if the administration was delayed up to 11 days after DFP, at which time some residual inhibition of NTE by DFP was still detectable.

From these experiments, we conclude that either protection from or promotion of OPIDP is caused by the same compounds when given in combination with a neuropathic OP. Both effects require NTE inhibition and the outcome depends on the sequence of dosing. The time–course and dose–response relationships of promotion also suggest that OPIDP initiation requires about 30% of NTE to be inhibited by DFP regardless of how inhibition is reached: either as residual inhibition several days after dosing or shortly after a smaller dose. The aging reaction might not be as relevant, at least quantitatively, in the initiation of OPIDP as it was thought. In our experiments low levels of inhibited/aged NTE complex (40–50%) are enough to cause OPIDP when promotion is provided. On the contrary when 30–50% of NTE is phosphorylated/aged after administration of a protective compound, no OPIDP develops (Johnson and Lauwerys, 1969; Johnson, 1982).

The rapid clearance of DFP and PMSF and the experimental design (they were given 24

TABLE 4

TIME COURSE OF OPIDP PROMOTION

Day of PMSF dosing (120 mg/kg sc) after DFP (0.5 mg/kg sc)	Percentage of NTE inhibition[a]	Clinical score[b]
No PMSF	79 ± 7 (8)	0.8 ± 1.2 (25)
3	69 ± 3 (3)	6.8 ± 1.8 (5)[c]
6	55 ± 11 (3)	6.0 ± 1.7 (5)[c]
11	32 ± 2 (3)	4.6 ± 2.3 (5)[c]
15	20, 16	1.8 ± 2.1 (5)
22	ND	1.0 ± 1.0 (3)

[a] Brain NTE activity was measured in paired dosed birds at the time of PMSF challenge to test birds. The percentage of inhibition was calculated from concurrent controls, one for each time point. Mean ± SD (*n*). ND, not done. All data were within the range of historical values for NTE activity.

[b] Mean ± SD (*n*) of maximal scores reached within 5 weeks after initial dosing.

[c] Different from birds treated with DFP only. $p < 0.05$, Mann–Whitney test modified for large samples.

TABLE 5

EFFECTS OF PMSF ON BIRDS PREVIOUSLY PROTECTED WITH PMSF FROM THE NEUROPATIC EFFECTS OF HIGH DOSES OF DFP

First dosing[a] PMSF	Second dosing DFP	Third dosing PMSF	Ataxia score[b]
30 mg/kg sc	1.5 mg/kg sc	—	0, 0, 0, 0
30 mg/kg sc	1.5 mg/kg sc	120 mg/kg sc	1.2 ± 0.8 (5)
30 mg/kg sc	1.5 mg/kg sc	480 mg/kg sc	1.8 ± 0.8 (5)

[a] Treatments were given 24 hr apart.

[b] Evaluated 21 days after DFP. Mean ± SD (n) or individual animals. Groups were different (Kruskal–Wallis test $p < 0.05$).

hr apart) rule out pharmacokinetic interactions. Furthermore data on NTE inhibition, when the two compounds were given to hens either alone or in combination, indicate that the inhibitor had reached the target. A sensitization of NTE by its subneuropathic inhibition/aging (50–60%) can also be excluded by repeated dosing experiments (Lotti and Johnson, 1980). In fact, this subthreshold effect on NTE is not related to an increased susceptibility of animals to OPIDP. A pharmacological effect on NTE analogous to that of partial agonist (protective/promoter) and agonist (neuropathic) might be inferred. How-

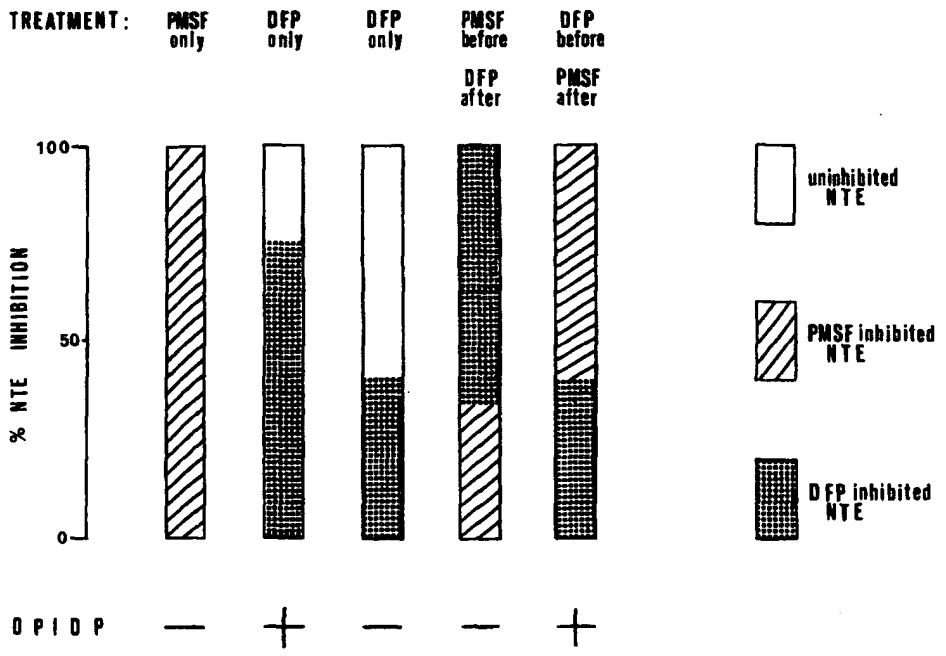

FIG. 1. Explicative summary of initiation, protection, and promotion of OPIDP. Relationships among dosing, NTE inhibition, and clinical response when DFP and PMSF are given alone or in combination.

ever, high doses of protective compounds have always failed to cause OPIDP (Johnson, 1974), even though more severe testing procedures might prove the contrary. Also the hypothesis that another site might be affected cannot be ruled out, but it is unlikely that another target shares similar structure–activity relationships with NTE.

These results might explain why OPIDP develops after dosing with racemic EPN [*O*-ethyl *O*-(4-nitrophenyl)phenylphosphonothioate] when it causes high inhibition of NTE but lower than threshold aging (Johnson and Read, 1987). Studies with resolved optical isomers showed that the L-(−) was neuropathic because of fast aging, whereas the D-(+) was nonneuropathic and protective because of slow aging of the inhibitor–NTE complex. In racemic mixtures the D-(+) isomer may have promoted OPIDP with lower than threshold aging of L-(−)-inhibited NTE.

Similar effects have been reported by Pope and Padilla (1989) when mipafox (5.0 mg/kg iv) was used as "initiator" and PMSF (60 mg/kg sc) as "potentiator." Their conclusion, however, differs from ours because they concluded that only when NTE is inhibited above threshold by the "initiator" will OPIDP potentiation occur.

The observation of protection from and promotion of OPIDP by the same compound may be relevant to the understanding of the physiology of target axons and also of the mode of action of neurotoxicants other than OPs. At the present time, however, these results have important implications for the toxicity testing of esterase inhibitors. Since several of these chemicals are used as pesticides and industrial chemicals, it was suggested (Johnson, 1980) to exploit this mechanistic knowledge to assess hazardous levels of exposure (OECD, 1983; U.S. Federal Register, 1985). While phosphinates and sulfonates do not have practical uses, carbamates do. Several publications state that compounds such as carbamates have no neuropathic potential and therefore should not be tested for OPIDP (Johnson, 1980; FAO/WHO, 1985; Lotti, 1989, 1990). However, carbamates which inhibit NTE promote OPIDP, and since mixed exposures to such potentially neuropathic, protective/promoting and racemic NTE inhibitors might occur, the perspective of their risk assessment must change.

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